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Original Article

Mature gonadal histology and gametogenesis of the tortoise beetle *Aspidimorpha sanctaecrucis* (Fabricius, 1792) (Coleoptera: Cassidinae: Chrysomelidae): Histological observation

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Abstract

The gonadal structure of insects has been well reported but there is a lack of such information from tortoise beetles that are an economically important insect. In this study, the gonadal structure and differentiating stage of gametogenesis of tortoise beetles *Aspidomorpha sanctaecrucis* are examined using the histological technique. At the level of light microscopy, our results revealed that the *A. sanctaecrucis* male reproductive system has a pair of testicular structures that consist of six seminiferous tubules. All zones contained various stages of spermatogenesis and could be divided into five stages depending on the shape and histological organization, particularly chromatin organization, which was divided into spermatogonia, primary spermatocytes with 8 sub-steps (leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase, anaphase, and telophase), secondary spermatocytes, spermatids (two sub-steps), and spermatozoa. A pair of ovaries was observed in the female *A. sanctaecrucis*. Each ovary is considered to be a meroistic ovariole that exists with nurse cells. The differentiating oogenic stages including previtellogenic and vitellogenic stages were observed. Our study provides baseline data that will be useful for future work involved in the ultrastructure and physiology of the reproductive tissue in tortoise beetles, especially the differential characterizations of the seminiferous tubule and ovarian type in beetles.

Keywords: germ cell, tortoise beetle, microanatomy, reproductive system

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1. Introduction

The reproductive systems of insects have received renewed interest because it is important to understand an insect's biology and physiology that have important implications such as biological control and the outbreak of an insect pest. The reproductive structures in insects, such as the shield bug (Özyurt, Candan, & Suludere, 2014; Özyurt, Candan, Suludere, & Amutkan, 2013), blister beetle (Gerber, Chltrc, & Rempel, 1971), and dock bug (Karakaya, Özyurt, Candan, & Suludere, 2012) have been well studied and provided important information on the anatomy, histology, and physiology of insects. Light microscopy revealed that the reproductive tissue of insects consists of two distinct parts, i.e. gonadal tissue and reproductive duct, based on the basic tissue and cell types in each region (Drecktrah, 1966; Özyurt *et al.,* 2013). Studies revealed that a pair of gonadal organs and a reproductive duct formed the reproduction system of insects such as in the *Nezara viridula* (Hemiptera: Pentatomidae) (Linnaeus, 1758; Pendergrast *et al.*, 1956; Ramamurty, 1969), *Ostrinia nubilalis* (Lepidoptera: Crambidae) (Drecktrah, 1966; Hübner, 1796), *Lytta nuttalli* (Coleoptera: Meloidae) Say 1824 (Gerber, Chltrc, & Rempel, 1971), *Abedus ovatus* (Stål, 1862) (Hemiptera: Belostomatidae) (Lalitha, Shyamasundari, & Hanumantha, 1997), *Coreus marginatus* (Linnaeus, 1758) (Heteroptera: Coreidae) (Karakaya *et al.*, 2012), *Graphosoma lineatum* (Linnaeus, 1758) (Hemiptera: Pentatomidae) (Özyurt *et al.*, 2013; Özyurt *et al.*, 2014), and *Catopsilia Pomona* Fabricius, 1775 (Lepidoptera: Pieridae) (Tongjeen *et al.*, 2014). Although the histological structures of the reproductive systems have been widely described in insects, a study on tortoise beetles has not been performed.

One of the most important tortoise beetles is the *A. sanctaecrucis* which belongs to the Coccinellidae family. This insect was chosen for this study because it is considered to be one of the most harmful insects that destroy plants, and it is an agricultural pest. Therefore, obtaining key information on the gonadal structures and gametogenic stages of *A. sanctaecrucis* in histological images is necessary.

2. Materials and Methods

Male (n=5) and female (n=5) tortoise beetles, *A. sanctaecrucis* were obtained from infected morning glory (*Ipomea aquatica*) which is an economically important vegetable in Southeast Asia. The mean length of the 10 beetles was 0.93±0.31 cm. The beetles were collected during the rainy season (October–December 2016) at Chawang District, Nakhon Si Thammarat Province, Thailand (8°28’ 10"N, 99°29’45"E). The tortoise beetles were euthanized by rapid cooling shock (Wilson, Bunte, & Carty, 2009) and were intermediately fixed with Davidson's fixative solution for ~48 h for histological investigation. Under histological investigation, the reproductive tissues were processed under standard histological techniques (Presnell & Schreibman, 2013; Suvarna, Layton, & Bancroft, 2013). All reproductive tissue blocks were cut at a thickness of 4 µm and routinely stained with Harris's hematoxylin and eosin and Masson's Trichrome (Presnell & Schreibman, 2013; Suvarna *et al.*, 2013). Histology of the gonadal structures and gametogenesis of the *A. sanctaecrucis* were determined and photographed under a light microscope (10x and 40x; LM TE2000-Ua).

Considering the lack of gonadal histological information of tortoise beetles, the aim was to be the first study to present data on the reproductive information of *A. sanctaecrucis* in both histological images and schematic diagrams.

3. Results and Discussion

3.1 Testicular histology and spermatogenesis

An important feature of the male *A. sanctaecrucis* was a pair of testis that consisted of six seminiferous tubules in the abdominal region (Figure 1A). The length of each tubule was about 800–850 µm. The vas efferent duct was jointed with the seminiferous tubule. The size of vas efferent duct was ~100 µm. It was lined by a simple high columnar epithelium. Each epithelial cell had a basophilic nucleus and was surrounded by a thin muscular layer (Figure 1B).

Histologically, the testis is prominently surrounded by a capsule comprised of a thick, loose connective tissue layer and a few smooth muscle layers that support the germinal cells. Each seminiferous tubule is covered by a thin layer of connective tissue (Figure 1C). The structure of a seminiferous tubule is divided into two zones, the vitellarium and germarium (Figure 1A) with differential development of the male germ cells. At high magnification, the vitellarium is distinctly seen at the outer rim of the tubule where a group of spermatogonia are arranged systematically. The seminiferous tubule is surrounded by a spherical cluster of spermatocytes. The seminiferous tubules (testis folicules) can be classified into three zones: growth; maturation; and transformation (Figure 1A). The zone of growth is composed of the spermatocytes under mitosis, which differs from spermatogonia. The maturation zone is found at the spermatids and is transferred under two meiotic divisions. The transformation zone contained spermatozoa. This feature is similar to other insects such as the *Podisus nigrispinus* (Lemos, Ramalho, Serrao, & Zanuncio, 2005) and *Dolycoris baccarum* (Özyurt *et al.*, 2012). Hereby, we confirmed that the seminiferous tubule of the *A. sanctaecrucis* is responsible for sperm production as similarly reported in *Graphosoma lineatum* (Özyurt *et al.*, 2013). The spermatogeneic stages of the tortoise beetle in the seminiferous tubules are classified into five stages depending on the shape and histological organization of chromatin organization. The stages are spermatogonia, primary spermatocytes with eight sub-steps (leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase, anaphase, and telophase), secondary spermatocyte, spermatids with two sub-steps and spermatozoa, as similarly reported in some invertebrates (Polijaroen, 2010; Sagi, Milner, & Cohen, 1988)

3.2 Spermatogonia

The spermatogonium is either of oval or spherical shape with a cell diameter ~10–12 µm. It contains a large centrally located nucleus, and the majority of the nuclei have the euchromatin with one or two nuclei (Figures 1B–1C, 1G–1H). The spermatogonium is also found in eosiophilic cytoplasm (data not shown).

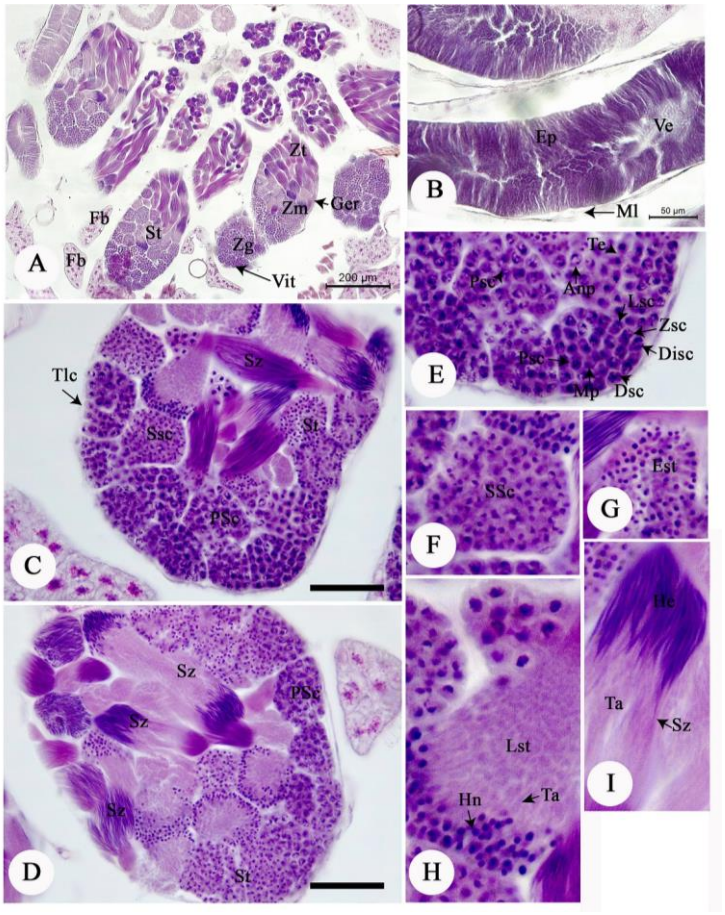

Figure 1. A–I. Light photomicrographs of the testicular structure and spermatogenesis in *Aspidomorpha sanctaecrucis*. Anp=anaphase, Dsc=diplotene primary spermatocyte, Disc=diakinesis, Ep=epithelium, Est=early spermatid, Fb=fat body, Ger=germarium, He=head, Hn=heterochromatin condensation, Lsc=leptotene, Lst=late spermatid, MI=thin muscular layer, Mp=metaphase, Psc=pachytene primary spermatocyte, SSc=secondary spermatocytes, St=seminiferous tubules, Sz=spermatozoa, Ta=tail, Te=telophase, Tlc=Tunica abuginea, Ve=vas efferent, Vit=vitellarium, Zg=growth, Zm=zone of maturation, Zt=zone of transformation, Zsc=zygotene.
Scale Bar: A, B, E, F, G, H, I = 20 µm; C, D = 50 µm.

3.3 Primary spermatocytes

The pachytene primary spermatocytes are cells with an oval or spherical shape with a mean cell diameter of ~10 µm (*A. sanctaecrucis*) (Figures 1C–1E). The primary spermatocytes have patchy heterochromatin throughout the basophilic nucleus. Hence, the structure of the chromatin fiber and heterochromatin is used to classify the stages of the primary spermatocyte which can be divided into eight sub-steps. (1) The leptotene spermatocyte has a spherical shape of ~8 µm in diameter and contains small heterochromatin blocks throughout the nucleus (Figure 1E). (2) The zygotene spermatocyte has a round nucleus similar to the leptotene spermatocyte but it is bigger. The density of heterochromatin blocks with the synaptonemal complexes was detected in the middle of the nucleus (Figure 1E). (3) The pachytene spermatocyte was still seen as in the previous steps but its large blocks or cords of heterochromatin were observed at the inner nuclear envelope (Figure 1E). (4) The diplotene spermatocyte was smaller than a pachytene spermatocyte (~7 µm in diameter). The oval nucleus contained a large chromatin block. It was arranged along the nuclear membrane and also seen as a cart-wheel pattern. (5) The diakinesis spermatocyte was a smaller cell compared to the diplotene spermatocyte (Figure 1E). The major characteristics of this stage were identified by the large pieces and almost fully formed chromosomes. At this step, the nuclear membrane had disappeared. (6) The metaphase spermatocyte was completely condensed with the chromatin and arranged along the equatorial region (Figure 1E). No nuclear membrane was seen in this stage. (7) The chromatin of anaphase spermatocyte moved to opposite poles of the cell (Figure 1E). (8) The final sub-step is the telophase spermatocyte where the cell membrane separates into two daughter cells.

3.4 Secondary spermatocytes (SSc)

The secondary spermatocyte appeared under the first meiotic division from primary spermatocyte but it was rarely observed because of rapid development. The cell size during this step was about 5 µm in diameter. The chromatin is of highly condensed cord-like structures (Figure 1F).

3.5 Spermatids

The differentiation of the spermatids occurs during spermiogenesis. Under the level of light microscopy, this stage could be divided into two sub-steps according to the chromatin condensation: early and late spermatids (Figures 1G–1H). The early spermatid is oval in shape and the cell is smaller than the secondary spermatocytes (~4 µm in diameter). The chromatin of this stage was condensed into interconnecting thick cords throughout the nucleus. The late spermatid had completely condensed heterochromatin and the beginning of the tail (Figure 1H).

3.6 Spermatozoa

Under the level of light microscopy, the mature spermatozoon had two distinct regions: head region and tail region (flagellum). Based on our observation, the head of the spermatozoon was an elongated shape (about 15–20 µm) and thin (Figure 1I).

3.7 Ovarian histology and oogenesis

A pair of ovaries of *A. sanctaecrucis* was observed. Each ovary contained several ovarioles (Figures 2A–2B). All ovarioles were surrounded by a peritoneal membrane (or sheath). Histological studies of the ovarian duct showed jointed ovaries and indicated that the ovarian duct was covered by a simple columnar epithelium (Figure 2C). The function of this epithelium is related to the production of a secretion with composting of carbohydrates and proteins which supports the production of the egg membrane (Gerber *et al.*, 1971). Both circular muscular and longitudinal muscular layers were also surrounded by this duct (Figure 2C).

The ovary is embedded by different stages of oocytes (Figure 2D) which was also considered to be a meroistic telotrophic type due to the presence of nurse cells in the terminal filament (Figure 1A). This type was similarly reported in *Perillus bioculatus* (Adams, 2000), *Perillus bioculatus* (Adams, 2001), *Podisus maculiventris* (Wittmeyer, Coudron, & Adams, 2001), and *Brontocoris tabidu* (Lemos *et al.*, 2005). The nurse cell is normally seen in the terminal filament (Figures 1E–1F). The function of the nurse cell involves supporting the nutrition for the oocyte (Klowden, 2007). Two distinct zones were divided according to histological features that included the germarium and vitellarium. It was similarly observed in coleopteran species (Buning, 2006).

3.8 Germarium

The oogonia were mainly contained in this zone and were mitotically processed; however, this stage is difficult to clarify under light microscopy. Ultrastructural observation is required to support this problem.

3.9 Vitellarium

This zone contains the differentiated oocytes in the mature ovaries including previtellogenic and vitellogenic stages in a linear arrangement (Figures 3A–3F).

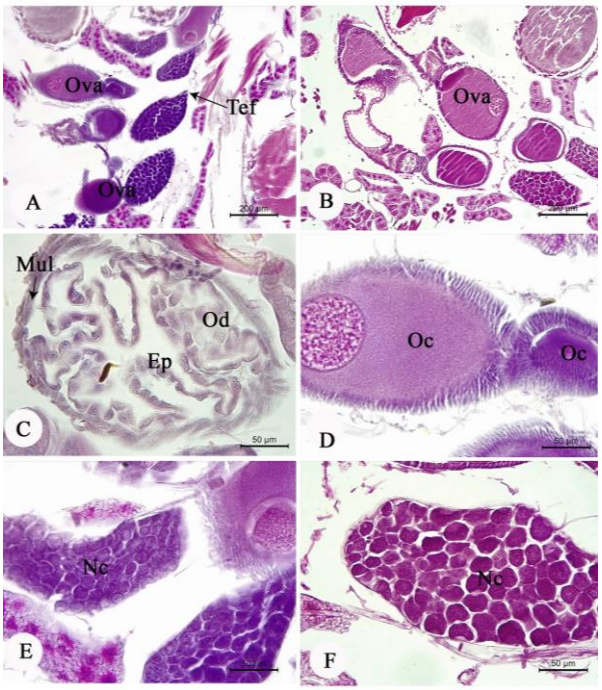

Figure 2. Figure 2A–F. Light photomicrographs of the ovarian structure and different stages of oocytes (Oc) in *Aspidomorpha sanctaecrucis*. Ep=epithelial layer, Mul=muscular layer, Nc=nurse cells, Od=ovarian duct, Ova=ovarioles.

The oval shape of the previtellogenic oocyte was observed to be about 300 µm in diameter (Figures 3A–3B). It contained a central nucleus of about 70 µm in diameter containing large heterochromatin blocks (Figure 3B). No nucleoli were detectable close to the nuclear membrane. The strong basophilic staining of the ooplasm also appeared (Figures 1A–3B). At the same time, a single layer of the elongated cuboidal follicle cells surrounding the oocyte was seen (Figures 3A–3D). At the end of this stage, the nucleus was of irregular shape and had obviously decreased in size to about 10 µm. On the other hand the increased follicular cell layer was noted as simple high columnar cells (Figure 3D).

The size of the vitellogenic oocyte had obviously increased to about 450–500 µm compared to the previous stages (Figures 1E–1F). Association to the major characterization showed that the large yolk granules were initially distributed, which were positively stained as a deep acidophilic stain in the ooplasm (Figure 1F). The follicular cells remained as a single layer; however, it changed to a low cuboidal cell type (Figure 3F).

4. Conclusions

Although information of the gonadal structure and gametogenesis from our observation is basic information, it was put forward to understand the histopathology/status of reproductive histology. We hope to follow this initial research and explore the reproductive system with relation to cellular association patterns, the requirements for yolk accumulation, the involvement of gonad-inhibiting hormones and the neurotransmitters, dopamine, and serotonin, which are known to be involved in the regulation of gonadal maturation.

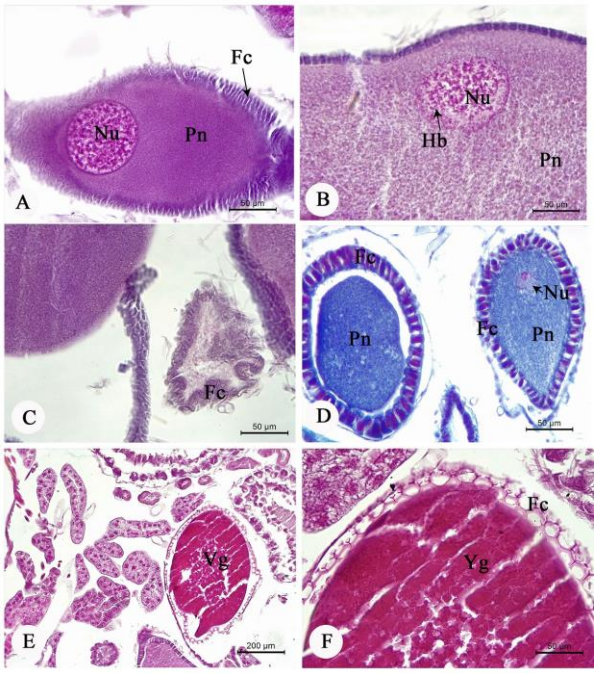

Figure 3. Figure 3A–F. Light photomicrographs of the different stages of oocytes in *Aspidomorpha sanctaecrucis*. Ep=epithelial layer, Fc=follicle cell, Hb=large hetero-chromatin block, Nu=nucleus, Pn=previtellogenic stage, Vg=vitellogenic stage, Yg=yolk granule.

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